Deletion of a Predicted Sensory Box Gene in *Shewanella oneidensis* MR-1 Causes Pleiotropic Phenotypes

A. Sundararajan[1], J. Kurowski1, A. Klonowska1, T. Yan2, D. M. Klingman2, M. Duley1, J. Zhou3, M. W. Fields1

[1]Department of Microbiology, Miami University, Oxford, OH, [2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, [3]Electron Microscope Facility, Miami University, Oxford, OH, [4]Institute for Environmental Genomics, University of Oklahoma, Norman, OK

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ABSTRACT. *Shewanella oneidensis* MR-1 can utilize a variety of electron donors and acceptors for energy, including oxygen and heavy metals. Understanding the physiological responses of MR-1 to environmental stresses is important in assessing the potential impact of such perturbations on metal-reducing activity. Here the possible physiological roles of a conserved hypothetical protein, SO3389 were characterized. The ORF contains PAS, PAC, EAL and GGDEF domains, and these domains have been implicated in multiple phenotypes. The exact physiological role(s) of proteins that contain these domains, however, have not been fully established. In addition, the possible role of a protein with this domain composition and architecture has not been previously described. Initial studies revealed that the mutant was impaired in motility and biofilm formation when grown aerobically compared to wild-type. The mutant lagged for 35 - 40 h when transferred from aerobic to anoxic medium, but growth rate was similar to wild-type once growth was initiated. Interestingly, when the mutant was transferred from anoxic growth, the subsequent lag was diminished. However, motility and biofilm formation were still impaired. This suggested that the mutant was affected in transitions from aerobic to anoxic conditions, motility, and biofilm. However, the phenotypes could be decoupled in subsequent transfers. In order to determine the cytochrome-c content of wild-type and mutant cells, spheroplast- and periplasmic-fractions were obtained. When the fractions from aerobic or anoxic grown cells were compared, some minor differences were observed. However, when cells were harvested during the transition from aerobic to anoxic conditions, major differences were observed in the spheroplast fraction, most noticeably for polypeptides of 57, 33, and 20 kDa. These results indicated that △SO3389 was also affected in c-type cytochrome content. This is the first report of a multi-domain PAS protein involved in biofilm formation. The data suggested that O2 may be a major signal that is sensed by SO3389, but further work is needed to elucidate the respective signal(s) and the mechanism(s) of signal transduction.

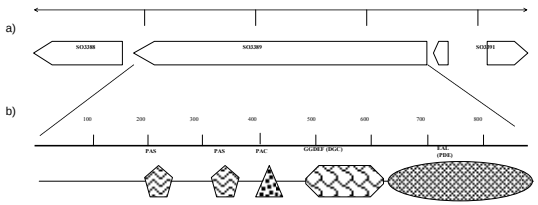
MATERIALS and METHODS. *S. oneidensis* MR-1 and SO3389 were grown in HBa Minimal Media containing 3mM PIPES, 7.5mM NaOH, 28.04mM NH_4Cl, 1.34mM KCl, 4.35mM NaH_2PO_4, 1.5mM Na_2SO_4, 10mL/L Minerals, 10mL/L Vitamins, 10mL/L Amino Acids (L-glutamate, L-arginine, D-L-serine), lactate at 90mM concentration (when grown aerobically), or lactate and fumurate at 120mM concentration each (when cells grown anaerobically). After autoclaving, 0.68mM $CaCl_2$ and 0.01mM Fe-NTA were added (pH 7.0). Aerobic cultures were grown in Erlenmeyer shake flasks with 50mL of media at 30°C and 150 rpm. Anaerobic cultures were grown in sealed 10mL flasks at 30°C with N_2 in the head space. At log phase, aerobic cultures were transferred to anaerobic medium at an O.D600 <0.03-0.06.

Motility analysis. 0.01mL of aerobic precultures of WT and SO3389 were spot inoculated on 0.3% and 1.0% agar HBa Minimal Media plates and grown at 30°C. Cytochromes were qualitatively assessed by heme-staining (using TMBZ staining method)

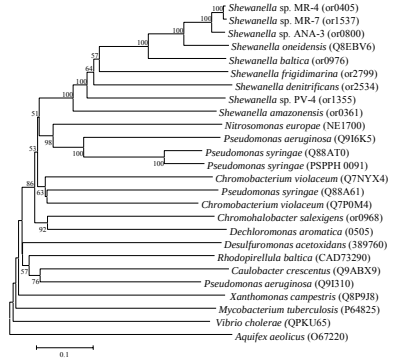
Biofilm quantification. Biofilms of WT and Δ3389 were quantified using crystal violet. Protein levels of the WT and Δ3389 grown in aerobic conditions were estimated by Lowry's method.

Excetracellular carbohydrate quantification. Extracellular carbohydrate was measured using calcoflour white, cells were stained using acridine orange.

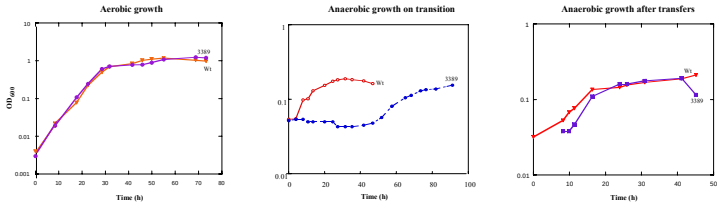
Reductase activity analysis. Reductase activities were detected as a clear band of oxidized methyl viologen after addition of fumarate and iron citrate to gels stained with blue, reduced form of methyl viologen.



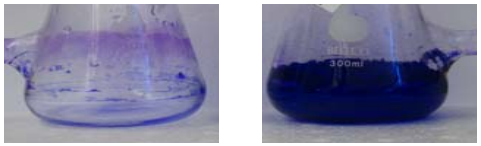
Domain architecture of ORF SO3389 (a) SO3389 and flanking genes, SO3388 (RNA helicase) and SO3390 (hypothetical protein). (b) Location of the domains PAS, PAC, GGDEF, EAL in ORF SO3389. The domains were predicted with SMART v4.0 (smart. Embl-heidelberg.de/smart/show_motifs.pl)



Phylogenetic relationship between the protein SO3389 and related sequences in sequenced genomes. SO3389 clustered with related genes in other *Shewanella* sp and other microorganisms.

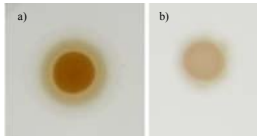


MR-1 cells were grown in HBa minimal medium with fumarate and lactate as electron acceptor and electron donor, respectively. For aerobic growth, cultures were grown in shake flasks (50 ml) at 150 rpm. The cell suspensions (wt and Δ3389) did not differ significantly in growth rate or final yields (planktonic). At log phase, cells were inoculated in anaerobic medium to an OD_{600} of 0.07. △3389 lagged for approximately 35-40 h compared to WT but final biomass yields were similar. Hence, the mutant could grow anaerobically, but was affected in the transition from aerobic to anaerobic conditions. Once the mutant grew anaerobically, subsequent transfers did not lag.



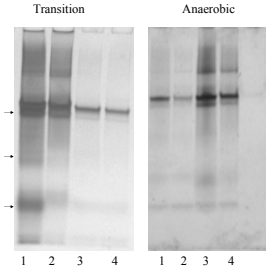
	Protein (μg/μL)
WT Biofilm	1.338
WT Pellet	5.890
△0550 Biofilm	0.307
△0550 Pellet	5.455
WT Total	7.228
△0550 Total	5.762

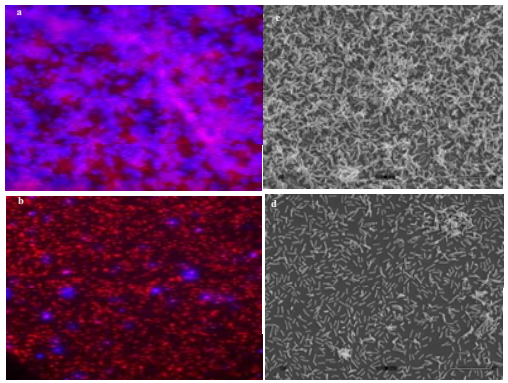
When cells were cultivated in shake flasks, WT cells formed thick biofilms in 2 to 3 days but △3389 cells did not. Biofilm was quantified by crystal violet staining. When protein levels were measured directly, the planktonic fractions did not differ significantly (5.9 vs. 5.5 ug/ul), but the WT biofilm fraction had 4.4-fold more protein than the mutant biofilm.



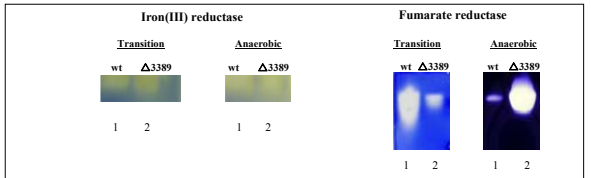
A colony of wild-type (**a**) or △SO3389 (**b**) cells on 0.3% motility agar inoculated from exponential phase aerobic cultures.

SDS-PAGE gels of spheroplast (lanes 1 and 2) and periplasmic-shock (lanes 3 and 4) fractions of wild-type and △SO3389 cells stained for c-type cytochromes. Equal amounts of total protein for wild-type (lanes 1 and 3) and △SO3389 (lanes 2 and 4) were loaded per well, and molecular weight markers were as follows (kDa): 150, 100, 75, 50, 35, 25, and 15. Differences observed at 20, 33 and 57 kDa molecular weights.



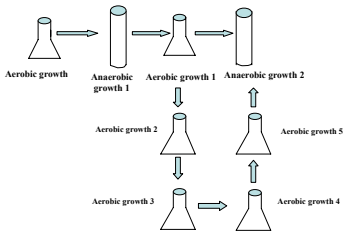


Scanning electron micrographs of wild-type (**c**) and △SO3389 (**d**) biofilms grown in defined, minimal medium with lactate for 60 h. Epifluorescent microscopy of wild-type (**a**) and △SO3389 (**b**) biofilms grown in defined, minimal medium with lactate for 60 h. Acridine orange was used to stain cells (red) and calcofluor white was used to detect extracellular carbohydrate (blue).



Native SDS-PAGE gels to detect iron reductase and fumarate reductase activities in WT and △SO3389 at transition and anaerobic phases of growth. Gels stained with reduced methyl viologen. Equal amounts of total proteins were loaded per well.

Schematic representing sequential transfers to aerobic medium and subsequent growth in anoxic medium. At every stage, motility and biofilm formation was tested and quantified.



Motility assay (a) and biofilm quantification (b) after four successive aerobic transfers. Δ3389 showed impaired motility and biofilm formation at every subsequent transfer. The WT was 2-fold more motile compared to the mutant and showed at least 5-fold more biofilm. However, the mutant did not lag after being transferred from aerobic to anoxic conditions. These results suggested that the mutant had overcome the loss of SO3389 but that the phenotypes were decoupled from one another. A possible explanation could be cross-talk between signal transduction pathways, but future work will be needed.

a)

Transfer #	Diameter (cm)	WT	Δ3389	Diameter in cms.
1)	1.9±0.3			1.2±0.2
2)	1.8±0.2			1.3±0.3
3)	2.0±0.2			1.0±0.2
4)	1.9±0.2			0.8±.02

b)

Transfer#	WT	Δ3389
1)	0.459	4.20
2)	0.478	5.90
3)	0.811	4.13
4)	0.186	2.65

CONCLUSION

- WT and mutant have similar growth rate when grown aerobically. Anaerobically, the mutant lags for ~ 40 hrs. The lag was observed when grown in different electron acceptors. Data suggested that the mutant had an altered ability to sense anoxic conditions.
- The mutant showed impaired motility and biofilm formation under aerobic conditions. Motility and biofilm formation were still impaired for the mutant after four transfers even though the initial lag during anoxic growth was lost.
- WT cells showed increased extracellular carbohydrate compared to mutant when stained with calcafluor white.
- Iron reductase activity was unchanged for the mutant under both anaerobic or transition growth conditions. Similar results were observed for DMSO and nitrate. Fumarate reductase activity was lower in the mutant cells but up-expressed under anaerobic conditions compared to wild-type cells.
- Minor differences were observed between WT and mutant cells under anaerobic conditions via 1-D SDA-PAGE. Significant differences were observed in cytochrome c polypeptide profiles in the transition phase between WT and mutant cells (57, 33, 20 kDa polypeptides).

VIMSS Virtual Institute for Microbial Stress and Survival

ESPP Environmental Stress Pathway Project